NMDA receptor activity downregulates KCC2 resulting in depolarizing $GABA_A$ receptor mediated currents

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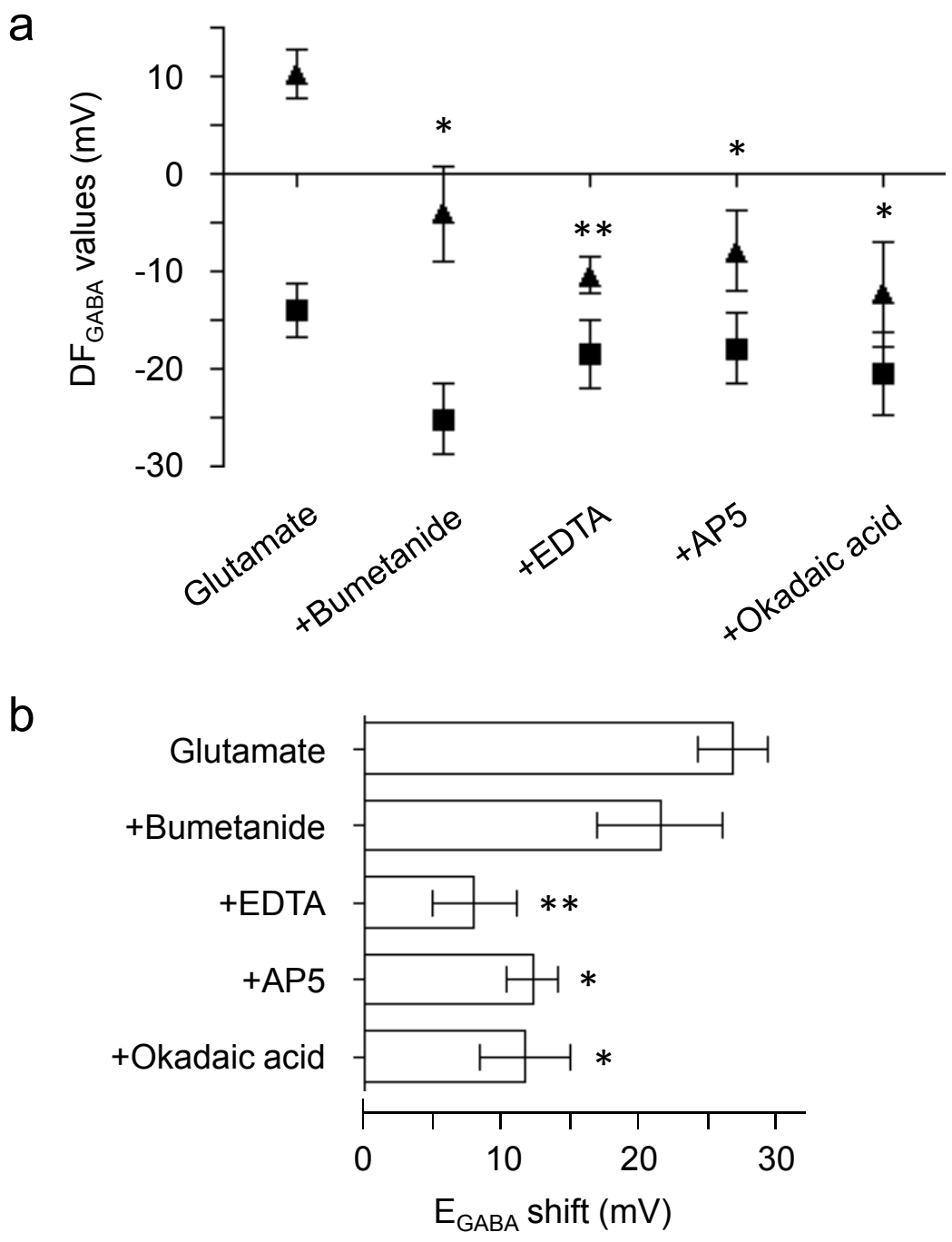
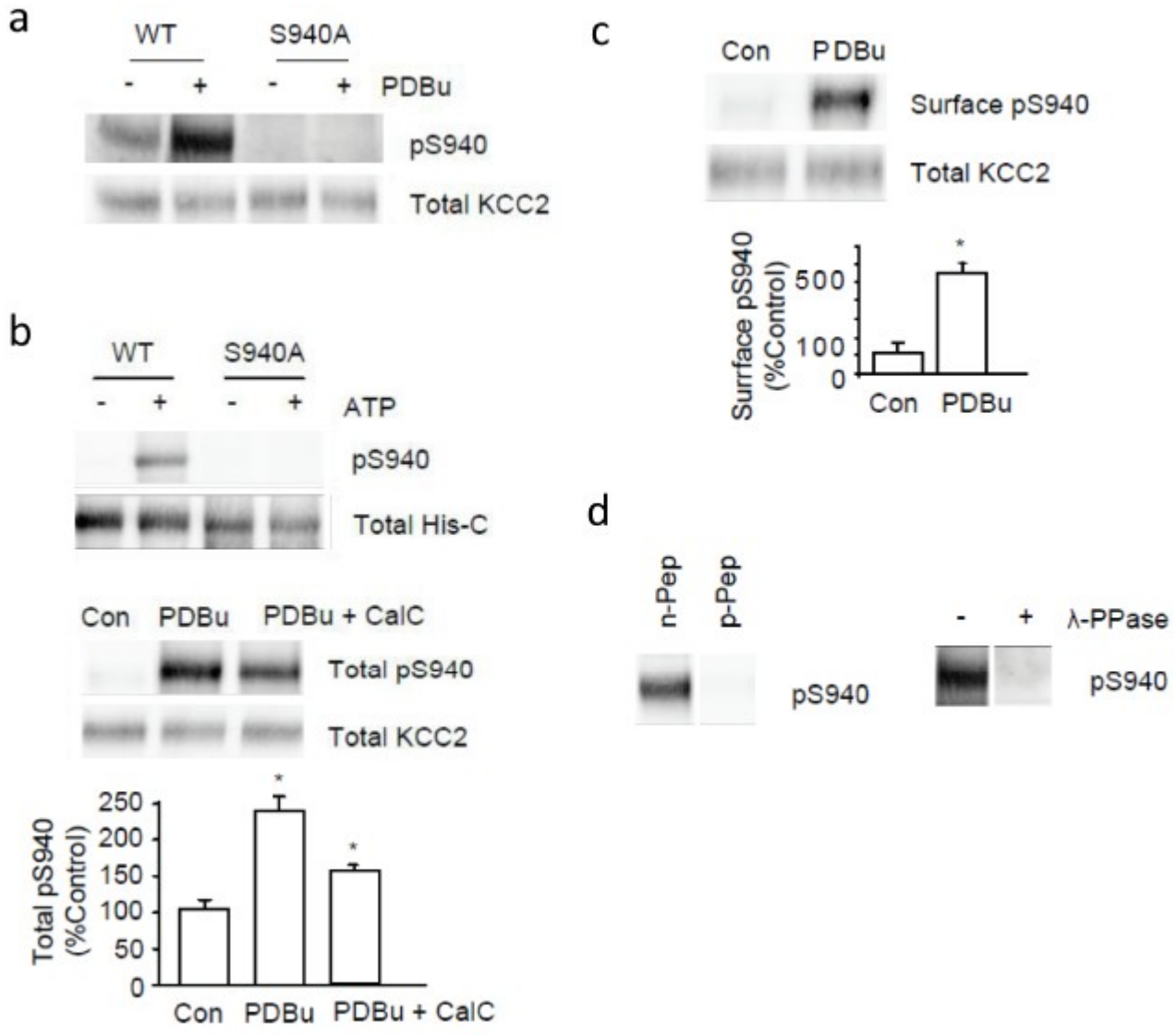
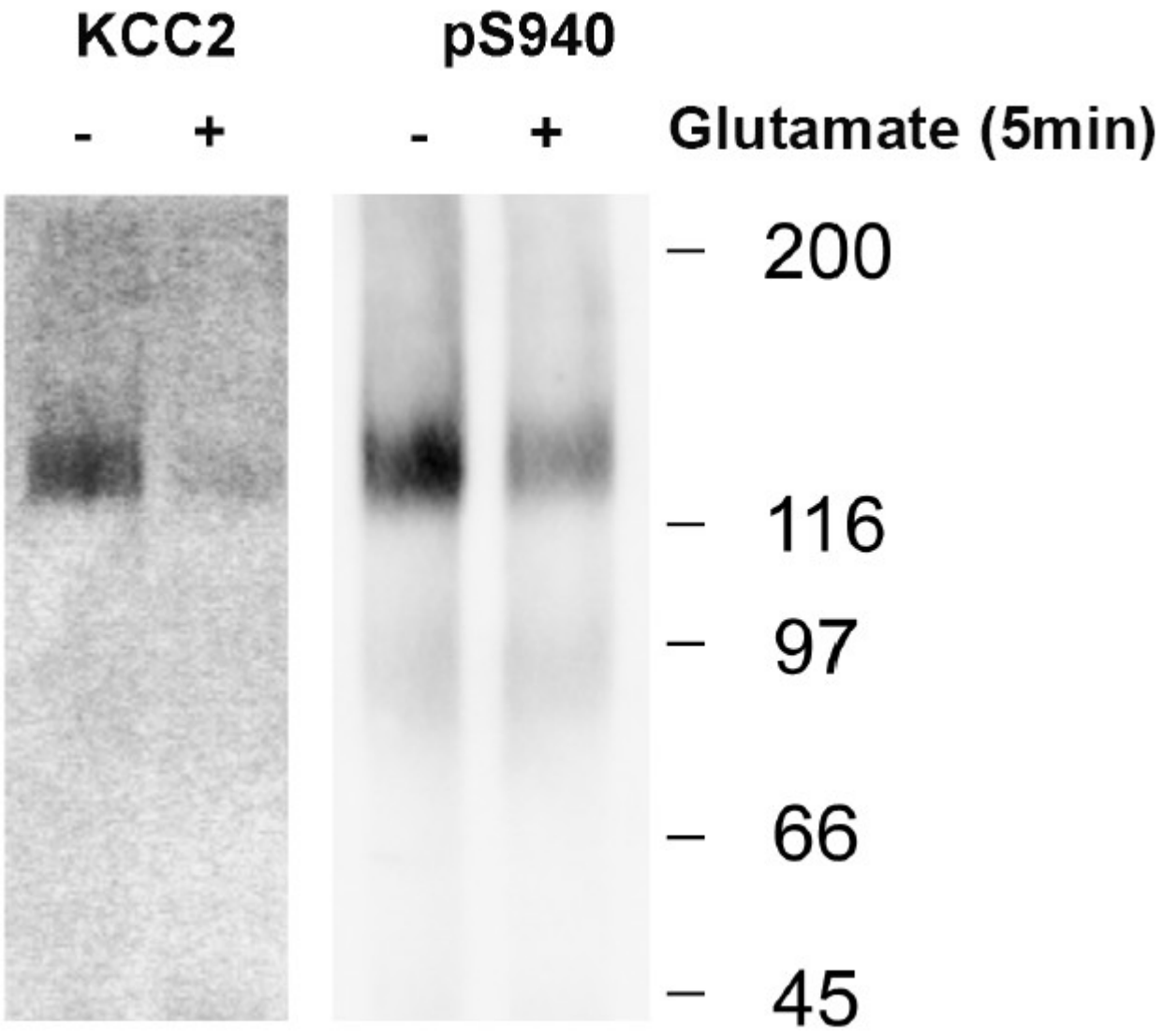


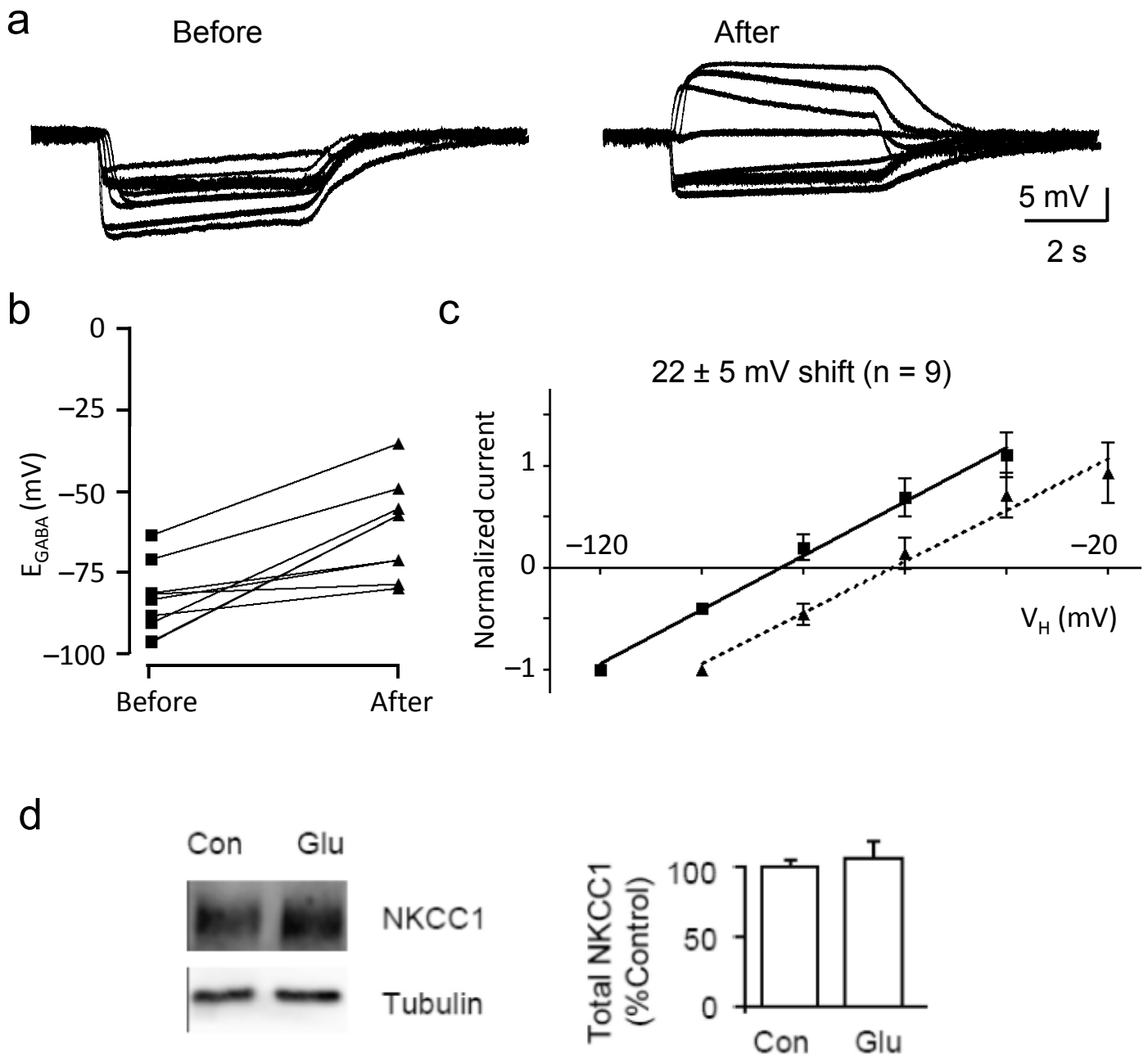
Supplementary Figure 1. Glutamate caused shifts in DF_{GABA} and E_{GABA} values. A, The calculated driving force on GABA-activated currents (E_{GABA} – E_M) was plotted before (squares) and after (triangles) glutamate application. The data was obtained with glutamate in the absence or presence of the indicated compounds. Stars mark statistical significance only for data obtained after glutamate treatment. B, Graph represents the calculated shift in E_{GABA} (E_{GABA} after – E_{GABA} before) caused by glutamate applied alone or in the presence of the indicated compounds. Error bars represent the mean ± SEM. Statistical significance was assessed by one-way ANOVA with a *post hoc* Dunnett's test; (*) significance of $p < 0.05$, (**) $p < 0.01$ compared to glutamate alone.



Supplementary Figure 2. The pS940 antibody was specific for phosphorylated S940. A, Immunoblots of lysates obtained from HEK-293 cells transfected with either wild type (WT) or the Ser to Ala (S940A) KCC2 mutant in control conditions ± 100 nM PDBu. B-top, His-tagged fusion protein of C-terminal domain of KCC2 (His-C) was subject to an *in vitro* kinase assay in the absence or presence of ATP. Reaction products were resolved by SDS-PAGE and immunoblotting with pS940. Total His-C used in the reactions was determined by anti-KCC2 antibody on a separate blot with equal amounts of protein loaded. B-lower, Cultured neurons were treated with 100 nM calphostin C (CalC) 15 min before application of PDBu (1 μM) for 15 min. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting with pS940 and anti-KCC2 antibodies. Quantified results are shown in the lower panel. C, Cultured neurons were treated with 1 μM PDBu for 15 min before a biotinylation assay and cell lysis. The cell surface portion and whole cell lysates were analyzed by SDS-PAGE and immunoblotting with pS940 and anti-KCC2 antibodies, respectively. Quantified results are shown in the lower panel. D-left, Immunoblots of neuronal lysates using the pS940 antibody in the presence of either the non-phospho-peptide (n-Pep) or phospho-peptide (p-Pep). D-right, Immunoblots of neuronal lysates in the absence or presence of λ-phosphatase (λ-PPase) on the blots before incubation of pS940. (*) Indicates statistically significant differences relative to controls as assessed by an unpaired t-test ($p < 0.01$; $n = 3$). Error bars represent the mean ± SEM.



Supplementary Figure 3. Glutamate decreased the levels of total KCC2 protein and phosphorylation of S940. SDS-soluble extracts from cultured hippocampal neurons treated with or without glutamate (20 μM) were subjected to SDS-PAGE (6 % gel), transferred to a membrane and then immunoblotted with KCC2 (left) or pS940 (right) antibodies. These full length blots were visualized using a phospho-imager. The position of molecular mass markers are indicated at the far right (kDa).



Supplementary Figure 4. The loss of hyperpolarizing GABA responses was not dependent on NKCC1 activity. A-C, Bumetanide (10 μM) was applied to neurons throughout the experiment. A, Membrane potential responses to exogenous GABA (10 μM) application obtained before (left) and after (right) a 2 min glutamate (20 μM) treatment. B, Dot plot of the extrapolated E_{GABA} values obtained before and after glutamate treatment. Lines connect the E_{GABA} values obtained for each cell. C, I-V plot of normalized GABA-activated currents obtained before (squares, solid line) and after (triangles, dashed line) glutamate treatment. The calculated shift in E_{GABA} caused by glutamate is displayed above the I-V plot. D, Cultured neurons were treated with glutamate (20 μM) for 15 min prior to assessing changes in total protein levels by immunoblotting with anti-NKCC1 antibodies. Error bars represent the mean ± SEM.